PATENT
Docket No. GC560

DIRECTED EVOLUTION OF MICROORGANISMS

FIELD OF THE INVENTION

The present invention relates to methods for directing the evolution of microorganisms using mutator genes. Such methods provide a pool of microbial genetic diversity advantageous for industrial application, such as for the industrial production of heterologous proteins, such as hormones, growth factors and enzymes, and the biocatalytic production of chemicals, vitamins, amino acids and dyes.

BACKGROUND OF THE INVENTION

The industrial applicability of microorganisms is restricted by their physiological limits set by solvent, pH, various solutes, salts and temperature. Organic solvents are generally toxic to microorganisms even at low concentrations. The toxicity of solvents significantly limits use of microorganisms in industrial biotechnology for production of specialty chemicals and for bioremediation applications. Solvent molecules incorporate into bacterial membranes and disrupt membrane structure (Isken and Bont, 1998, Extremophiles 2(3): 229-238); (Pinkart and White, 1997, J. Bacteriol. 179(13): 4219-4226); (Ramos, Duque et al., 1997, " J. Biol. Chem. 272(7): 3887-3890); (Alexandre, Rousseaux et al., 1994, FEMS Microbiol, Lett, 124(1): 17-22); and Kieboom, Dennis et al., 1998 ,J. of Bacteriology 180(24): 6769-6772).
Classic strain improvement methods including UV and chemical mutagenesis have been applied for selection of more tolerant strains (Miller, J., "A Short Course In Bacterial Genetics," *Cold Spring Harbor Laboratory Press*, 1992). A number of studies have been dedicated to identification and isolation of solvent tolerant mutants among various bacterial strains. Spontaneous *E. coli* solvent tolerant mutants and mutants isolated in the process of 1-methyl-3-nitrosoguanidine (NTG) mutagenesis were obtained from strain K-12 (Aono, Aibe et al., 1991 Agric. Biol. Chem 55(7): 1935-1938). The mutants could grow in the presence of diphenylether, *n*-hexane, propylbenzene, cyclohexane, *n*-pentane, *p*-xylene. Various Pseudomonas strains were able to adapt and to grow in a toluene-water two-phase system (Inoue and

Horikoshi, 1989, Nature 338: 264-266), with *p*-xylene (Cruden, Wolfram et al., 1992, Appl. Environ. Microbiol. 58(9): 2723-2729), styrene and other organic solvents (Weber, Ooijkaas et al., 1993, Appl. Environ. Microbiol. 59(10): 3502-3504), (de Bont 1998, Trends in Biotechnology 16: 493-499). Yomano et al. isolated ethanol tolerant mutants which increased tolerance from 35g/l to 50g/l during 32 consequent transfers (Yomano, York et al., 1998, J. Ind. Microbiol. Biotechnol. 20(2): 132-138). High temperature evolution using E.coli has been disclosed in the art (Bennett, 1990, Nature, Vol. 346, 79-81) however the fitness gain was low as compared to the parent.

Strains of *E. coli* that carry mutations in one of the DNA repair pathways have been described which have a higher random mutation rate than that of typical wild type strains (see, Miller *supra*, pp.193-211). As reported by Degenen and Cox (*J. Bacteriol.*, 1974, Vol. 117, No. 2, pp.477-487), an *E. coli* strain carrying a mutD5 allele demonstrates from 100 to10,000 times the mutation rate of its wild type parent. Greener et al., "Strategies In Molecular Biology," 1994, Vol. 7, pp.32-34, disclosed a mutator strain that produces on average one mutation per 2000 bp after growth for about 30 doublings.

Microorganisms are used industrially to produce desired proteins, such as hormones, growth factors and enzymes and to produce chemicals, such as glycerol and 1,3 propanediol (WO 98/21340 published May 22, 1998 and U.S. Patent No. 5,686,276 issued November 11, 1997), vitamins, such as ascorbic acid intermediates (1985, Science 230:144-149), amino acids, and dyes, such as indigo (U.S. Patent No. 4520103, issued May 28, 1985). In spite of advances in the art, there remains a need to improve the microorganisms and methods for producing such desired proteins, chemicals, amino acids and dyes.

SUMMARY OF THE INVENTION

The present invention relates generally to methods for directing the evolution of a microoganism, that is for directing desired genetic change in a microorganism in response to conditions of selective pressure. In one aspect, the present invention relates to methods for evolving microorganisms to grow under extreme conditions, such as at high temperature, under conditions of pH extremes, in the presence of solvents, and in the presence of high salt. In another aspect, the present invention relates to methods for evolving a microorganism comprising at least one nucleic acid

encoding a desired protein or an enzyme in an enzymatic pathway to grow under desired conditions.

The present invention is based, in part, upon the finding that microrganisms such as wild-type E.coli and E.blattae, can be evolved into microorganisms capable of growing in the presence of high solvents, such as DMF and 1,3 propanediol, using methods described herein. The present invention is also based, in part, upon the finding that E.coli can be evolved into a microorganism capable of growing at elevated temperatures using methods described herein. The present invention is further based, in part, upon the identification of the optimal mutation rate for a microorganism and the discovery that the mutation rate can be controlled.

Accordingly, the present invention provides a method for preparing an evolved microorganism comprising the steps of culturing a microorganism comprising at least one heterologous mutator gene for at least 20 doublings under conditions suitable for selection of an evolved microorganism, wherein said heterologous mutator gene generates a mutation rate of at least about 5 fold to about 100,000 fold relative to wild type, and restoring said evolved microorganism to a wild type mutation rate. In one embodiment, the microorganism further comprises at least one introduced nucleic acid encoding a heterologous protein, said protein(s) including, but not limited to hormones, enzymes, growth factors. In another embodiment, the enzyme includes, but is not limited to hydrolases, such as protease, esterase, lipase, phenol oxidase, permease, amylase, pullulanase, cellulase, glucose isomerase, laccase and protein disulfide isomerase. The present invention encompasses genetic changes in the microorganism as well as changes in the introduced nucleic acid.

In yet a further embodiment, the microorganism further comprises introduced nucleic acid encoding at least one enzyme necessary for an enzymatic pathway. In one embodiment, the introduced nucleic acid is heterologous to the microorganism; in another, the introduced nucleic acid is homologous to the microorganism. In a further embodiment, the enzyme is a reductase or a dehydrogenase and said enzymatic pathway is for the production of ascorbic acid or ascorbic acid intermediates. In an additional embodiment, the enzyme is glycerol dehydratase or 1,3-propanediol dehydrogenase and said enzymatic pathway is for the production of 1,3 propanediol, 1,3 propanediol precursors or 1,3 propanediol derivatives. In another embodiment, the enzyme is glycerol-3-phosphate dehydrogenase or glycerol-3-phosphate phosphatase and said pathway is for the production of glycerol

and glycerol derivatives. In a further embodiment, the enzymatic pathway is for the production of amino acids, such as tryptophane or lysine or dyes, such as indigo.

In one embodiment of the present invention, the microorganism is cultured for between about 20 to about 100 doublings; in another embodiment, the microorganism is cultured for between about 100 to about 500 doublings; in yet another embodiment, the microorganism is cultured for between about 500 to about 2000 doublings and in a further embodiment, the microorganism is cultured for greater than 2000 doublings. In one embodiment, the mutator gene generates a mutation rate of at least about 5 fold to about 10,000 fold relative to wild type; in another embodiment, the mutator gene generates a mutation rate of a least about 5 fold to about 1000 fold and in another embodiment, the mutator gene generates a mutation rate of about 5 fold to about 100 fold over wild type.

In one embodiment, an evolved microorganism comprises from about 3 to about 1000 selected mutations in about 3 to about 500 genes and may further comprises from about 20 to about 100,000 neutral mutations. In one aspect, the mutations generated are non-specific and in another aspect, the mutations generated are specific.

In one embodiment of the present invention, the microorganism comprises a plasmid comprising the heterologous mutator gene and said step of restoring said evolved microorganism to a wild type mutation rate comprises curing the evolved microorganism of said plasmid. In another embodiment, the plasmid comprises a temperature sensitive origin of replication and the curing comprises growing the evolved microorganism at a restrictive temperature. In a further embodiment, the microorganism comprises at least one copy of the mutator gene in the chromosome and said step of restoring said evolved microorganism to a wild type mutation rate comprises excision or removal of said mutator gene from the host genome or the replacement of the mutator gene with a functional (non-mutator) allele of the same gene.

In one embodiment, the present invention comprises the use of at least one mutator gene to evolve a microorganism. In another embodiment, the mutator gene includes but is not limited to a mutD gene mutation, a mutT gene mutation, a mutY gene mutation, a mutM gene mutation, a mutH gene mutation, a mutL gene mutation, a mutS gene mutation or a mutU gene mutation and homologues of these DNA repair genes which have been mutated as long as the mutated gene has

impaired proofreading function. In a further embodiment, the mutator gene comprises at least one of the mutD mutations disclosed herein in Table I.

In one embodiment of the present invention, conditions suitable for selection include but are not limited to culturing said microorganism in the presence of at least one organic solvent, such as for example, alcohols, diols, hydrocarbon, mineral oil, mineral oil derived products, halogenated compounds and aromatic compounds; in the presence of high temperature, such as in the range of 42°-48° C; in the presence of high salt, and in the presence of extreme pH conditions, such as alkaline or acidic conditions.

The present invention encompasses methods for evolving gram positive and gram negative microorganisms as well as yeast, fungus and eucaryotic cells including hybridomas. In one embodiment, the gram negative microorganism includes members of *Enterobacteriaceae* and in another embodiment comprises *Eschericia* and in another embodiment comprises *E.coli* and *E.blatte*. In further embodiments of the present invention, the evolved microorganism includes E.coli having ATCC accession number _____ and E.blatte having ATCC accession number _____.

The present invention also provides expression vectors and host cells comprising a mutator gene and methods for producing such vectors and host cells.

SUB B1

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleic acid and amino acid sequence of the mutD gene. Illustrative examples of mutations of the mutD gene are provided.

Figure 2 provides the nucleic acid sequence for the enzyme 1,3-propanediol dehydrogenase (PDD).

Figure 3 provides the amino acid sequence for the enzyme 1,3-propanediol dehydrogenase (PDD).

Figure 4 provides a time course for E.coli cultures subjected to directed evolution and selection under elevated temperature.

Figure 5 - Glycerol fermentation of *E. blattae* at pH 7.0. Culture conditions are described in the text. Plate counts were by serial dilution and performed in triplicate on Luria agar plates. Substrate and 0products were measured by HPLC.

Figure 6 - Glycerol fermentation of *E. blattae* strain GEB031-4 at pH 7.0. Culture conditions are described in the text. Plate counts were by serial dilution and

performed in triplicate on Luria agar plates. Substrate and products were measured by HPLC.

DESCRIPTION OF THE MICROORGANISM DEPOSITS MADE UNDER THE BUDAPEST TREATY

Applicants have made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure:

Depositor Identification Reference	International Depository Designation	Date of Deposit
Escherichia coli MM294 derivative	ATCC	May 17, 1999
Escherichia blattae 33429 derivative	ATCC	May 17, 1999

DETAILED DESCRIPTION

Definitions

A mutation refers to any genetic change that occurs in the nucleic acid of a microorganism and may or may not reflect a phenotypic change within the microorganism. A mutation may comprise a single base pair change, deletion or insertion; a mutation may comprise a change, deletion or insertion in a large number of base pairs; a mutation may also comprise a change in a large region of DNA, such as through duplication or inversion.

When many possible different mutations in nucleic acid can give rise to a particular phenotype, the chance of mutation to that phenotype will be higher than in a situation where only a few types of mutations can give rise to a particular phenotype. As used herein the terms "wild-type mutation" and "spontaneous mutation" are used interchangeably. The rate of spontaneous mutation is defined as the probability of a mutation each time the genome is replicated or doubled. As used herein "mutation rate" is simultaneous with "frequency" and refers to the absolute number of mutations/doubling/base pair. As used herein, the term "relative rate" refers to the ratio of mutation rates of two strains, one of these is usually a wild type strain. Relative rate indicates how much more likely it is that a strain will undergo mutation as compared to the wild type strain. The frequency of spontaneous mutation of wild type E. coli (the E.coli genome has about 4.6×10^6 base pairs) is

about $5x10^{-10}$ mutations per base pair per doubling (see Drake, 1991). Doubling refers to the process of reproduction of at least part of a genome of an organism and usually involves reproduction by binary fission. As used herein, "doubling" encompasses the reproduction of nucleic acid within an microorganism achieved by any means.

As used herein, a "mutator strain" refers to a microorganism having a higher than naturally occurring rate of spontaneous mutation. As used herein, "mutator gene" refers to a DNA repair gene which comprises a mutation and which has impaired proof reading function. As used herein, the term "mutator plasmid" refers to a plasmid or expression vector or cassette comprising a mutator gene. Culturing a microorganism comprising a mutator gene will give rise to mutational events during genome replication. The present invention encompasses the use of any DNA repair genes comprising mutations as long as the mutated DNA repair gene is capable of introducing mutational events in a microorganisms genome or on a gene introduced into the microorganism. DNA repair genes include but are not limited to, mutD, mutT, mutY, mutM, mutH, mutL, mutS or mutU and homologues of these genes. A homologue as used herein refers to a functionally related DNA repair gene. In one embodiment, the mutator gene is a mutD gene (the epsilon subunit of DNA polymerase III, see Degnen et al., 1974, J. Bacteriol. 117:477-487) comprising mutations that provide an impaired proofreading function. In one embodiment disclosed herein, the mutD mutation is introduced into a microorganism on a plasmid. Illustrative embodiments of MutD mutations are disclosed herein in Table I. The mutD mutations impair proofreading function of the epsilon subunit of DNA polymerase III holoenzyme by significant decrease in the 3'-5' exonuclease activity (Takano et al., 1986, Mol. Gen. Genet. 205(1):9-13).

When referring to mutations or genetic changes in an evolved microorganism, "neutral mutation" refers to a mutation which has little or no measurable effect on the phenotype of an evolved strain under a given set of conditions. Examples of "neutral mutations" include, but are not limited to, silent mutations which do not lead to a change in the amino acid sequence of the encoded protein, mutations which affect proteins that are not essential for growth under a given set of culture conditions, and mutations in non-coding regions of the chromosome. In one illustrative embodiment herein, an E.coli strain evolved for high temperature was characterized as being auxotrophic for three amino acids (ie, were not able to grow in medium without Cys/Met, Asp/Asn, and Pro) indicating that there

were at least three neutral mutations in the E.coli in addition to the mutations associated with growth at high temperature. The term "selected mutation" as used herein refers to those mutations which are associated with a phenotype of an evolved strain under a given set of conditions. Being associated with means that the mutation is directly or indirectly responsible for the improved or altered phenotype.

When referring to mutations or genetic changes in a host cell or microorganism, nonspecific refers to the changes in the host cell genome which occur randomly throughout the genome and which potentially can affect all bases and includes frameshifts. Non-specific mutations encompass changes in single base pairs as well as changes in a large number of base pairs as well as changes in large regions of DNA. For example, in one embodiment, an evolved microorganism which has been exposed to a MutD gene comprising mutations that impair the proof reading function will comprise random mutations at a rate of about 5-1000 times over wild type. In one illustrative embodiment of the method using a mutD mutation, the evolved strain had at least 3 random mutations. The present invention encompasses any rate of mutations that provides the desired phenotype. When referring to genetic changes in a host cell, specific mutation refers to mutations which can be characterized or which comprise definable genetic changes, such as A:T to C:G transversion characteristic of mutT mutations; G:C to T:A transversion characteristic of mutY and mutM mutations; A:T to G:C and G:C to A:T transitions and frameshifts characteristic of mutH, mutL, mutS, uvrD (mutU) mutations; G:C to T:A transversions characteristic of the mutYmutM double mutation (Miller et al., A Short Course in Bacterial Genetics, a Laboratory Manual and Handbook for E.coli and Related Bacteria).

When referring to a mutator gene, "heterologous" means that the gene is introduced into the cell via recombinant methods and is preferably introduced on a plasmid. The mutator gene may also be introduced into the microorganism genome through recombinant techniques. The mutator gene introduced into the microorganism may be a mutation of a naturally occurring DNA repair gene in the cell or may be foreign to the host microorganism. Referring to nucleic acid as being "introduced" into a microorganism means that the nucleic acid is put into the microorganism using standard molecular biology techniques. An introduced nucleic acid may be the same or different than nucleic acid naturally occurring in the microorganism.

As used herein the term "restoring to wild type mutation rate" refers to the process whereby a mutator gene is removed from an evolved microorganism thereby restoring the wild-type mutation rates. The present invention encompasses any process for removing the mutator gene from an evolved organism and includes but is not limited to curing the organism of a resident plasmid comprising the mutator gene or by excising or otherwise removing the mutator gene from the host genome such that normal DNA repair function is restored. Curing refers to methods for producing cells which are free of a plasmid comprising the mutator gene. A microorganism can be cured of any resident plasmid using techniques known to the skilled artisan.

Detailed Description

One of the basic tenants of inheritance is that mutations occur randomly and then are selected by the environment. Mutations that happen to confer a selective advantage on the organism are preferentially passed on to future generations. The present invention relates to methods for directing desired genetic change in a microorganism, ie directing the evolution of a microorganism, by exposing the microorganism to a mutator gene, selecting for acquisition of desired characteristics in the evolved microorganism, and curing the microorganism of the mutator gene, or otherwise removing the mutator gene, such that wild type mutation rates are restored.

I. Uses of the Invention

In one aspect of the present invention, the methods are used to evolve a microorganism to grow under extreme conditions, such as in the presence of elevated temperature, high solvent, altered pH or in the presence of high salt. In another aspect of the present invention, the methods are used to evolve microorganisms which comprise introduced nucleic acid encoding a heterologous protein or at least one enzyme in an enzymatic, ie biocatalytic pathway. Such commercially important proteins include hormones, growth factors and enzymes. Illustrative biocatalytic pathways include those disclosed in U.S. Patent No. 5,686,276, issued November 11, 1997, for the production of 1,3-propanediol and in 1985, Science 230:144-149 for the production of ascorbic acid intermediates.

Methods of the present invention are especially advantageous for producing improved microorganisms used for the biocatalytic production of chemicals and vitamins where numerous catalytic events are taking place either concurrently or

sequentially within the host microorganism. In such complex biocatalytic systems, it is often difficult to identify the specific molecular events causing low yields, host toxicity or catalytic failures and therefore difficult if not impossible to understand which specific genetic events to alter in order to correct the deficiencies. The methods of the present invention provide the advantage of allowing the microorganism to make the required changes in response to selective pressure.

Additionally, the methods of the present invention provide an advantage for obtaining microorganisms comprising desired phenotypic traits associated with multiple genes, such as the ability of a microorganism to grow at elevated temperatures. The use of the mutator gene provides a means for producing genetic diversity and the simultaneous growth under conditions of selective pressure allows the microorganism to identify the specific genetic changes required for survival. The use of mutD gene mutations allows for very large diversity to be provided to the microorganism from which to select for the specific genetic changes that provide a growth advantage. Therefore, the methods disclosed herein avoid the limited diversity that is often produced with art methods that begin the directed evolution process with defined sets of genes. Furthermore, the methods disclosed herein eliminate additional screening steps often associated with art methods for producing genetic diversity. A further advantage of the present invention is that the methods can be applied to microorganisms which have not been sequenced and for which there may be limited information upon which to design genetic changes.

In illustrative embodiments disclosed herein, a mutated mutD gene residing on a plasmid was introduced via recombinant techniques into E.coli or E.blatte. The E.coli or E.blatte cell was then cultured under conditions suitable for growth for a time sufficient for at least 20 doublings and up to at least about 2000 doublings under conditions of selective pressure. In one example, E.coli was grown under conditions of increased temperature or in the presence of DMF and in another E.blattae was growth in the presence of solvent, such as DMF or 1,3 propanediol. As a result, E.coli was evolved into a microorganism capable of growing at temperatures up to about 48° C or in the presence of 80g/l DMF. E.coli evolved to grow at elevated temperatures also became auxotrophic for three amino acids, Cys/Met, Asp/Asn and Pro. E.blattae was evolved into a microorganism capable of growing anaerobically in the presence of at least 105 g/l 1,3-propanediol and which comprised genetic changes in at least one catalytic activity associated with 1,3 propanediol production, 1,3-propanediol dehydrogenase, shown in Figure 3.

Sub B3

The use of a plasmid comprising a mutator gene, ie, a mutator plasmid, can be used to control the mutation rate of a microorganism. As described under Section II below, plasmid constructs can be designed which provide reduced levels of expression of a mutator gene thereby providing a means for altering the ratio of naturally occurring DNA repair genes vs mutator genes. This provides a means for combining the advantage of mutD mutations (which results in random mutagenesis) with the advantages of the other known mutators (lower mutation frequency which leads to a lower burden on the cells). Additionally, plasmid constructs can be designed that allow for curing the evolved microorganism of the mutator gene, such as the use of a temperature sensitive origin, thereby allowing for a means for turning the mutation events off and on in the microorganism. For a gram positive microorganism, such as B.subtilis where the entire genome has been sequenced, the present invention could encompass the steps of deleting or mutating a DNA repair gene, evolving the Bacillus, and restoring the naturally occurring DNA repair system through recombination events. As disclosed herein, several members of Escherichia, such as E.coli and E.blattae have been subjected to the directed evolution methods. Illustrative examples of evolved E.coli and E.blattae have been deposited with the ATCC and have accession numbers, ________ and ________, respectively.

The methods of the present invention provide a means to accomplish long-term evolution of microorganisms. An E.coli strain comprising a plasmid comprising a mutD mutation was grown for >1000 doublings without a reduction in mutation rate. The present invention also provides a means for reducing the functional genome of an organism. A microorganism can be grown for many thousands of generations, such that only the genes which are essential would remain functional. Most of the other genes would carry random and inactivating mutations.

The present invention also provides a means for making non-pathogenic organisms. A pathogenic strain can be evolved into a mutator strain by introduction of a mutator gene and grown for extended periods of time. As a result many of the genes that are involved in pathogenicity would become inactivated and the strain would be safe to use.

The present invention also provides a means to streamline the metabolism of an organism. A strain which has an improved yield on nutrients or a reduced metabolic rate (maintenance metabolism) can be produced by methods disclosed herein. Such strains would be useful production strains for chemicals as well as

enzymes. The present invention provides a means for making microorganisms mutator strains by introducing a mutator gene, thereby protecting the microorganism's naturally occurring DNA repair genes from becoming mutator genes in response to selective pressure. That is, the introduction of the mutator plasmid into a microorganism whether via a plasmid or into the genome, protects the cells from developing a mutator phenotype in response to selective pressure.

II. Mutator genes and frequency of mutations

Mutator genes of the present invention include but are not limited to, mutations of the DNA repair genes mutD, mutT, mutY, mutM, mutH, mutL, mutS or mutU or their homologues in other microorganisms. A description of the DNA repair genes are disclosed in Miller, *supra*; mutD is disclosed in Maki et al., 1983, Proc. Natl. Acad. Sci., U.S.A. 80, 7137-7141 (GenBank accession number K00985.1 GI: 147678 and Figure 1); B. subtilis mutS and mutL are disclosed in Ginetti et al., 1996, Microbiology, Aug, 142 (Pt 8): 2021-9; Streptococcus pneumoniae hex B repair gene, mutL of Salmonella typhimurium and PMS1 of Saccharomyces cerevisiae are disclosed in Prudhomme et al., 1989, J. Bacteriology, Oct; 171 (10): 5332-8; Streptococcus pneumoniae hexA and mutS of Salmonella typhimurium and E.coli are disclosed in Priebe et al., J. Bacteriol, 1988, Jan; 170(1): 190-6 and Prudhomme et al., 1991, J. Bacteriol. Nov; 173(22): 7196-203; human mutS homologue, hMSH2, and human MutL homologue, hMLH1, are disclosed in Macdonald et al., 1998, Heptology, Jul 28(1):90-7; the mut-1 of Neurospora is disclosed in Dillon et al.,1994, Genetics, Sep 138(1):61-74 and yeast homologues of mutL and mutS are disclosed in WO 97/15657. The methods of the present invention comprises the use of at least one of the mutant DNA repair genes and may involve the use of more than one. It is preferred that a mutator gene be dominant to the wild type gene of the microorganism such that mutations are introduced into the genome of the microorganism. In a preferred embodiment, the mutator gene is a mutation of the mutD gene. The nucleic acid and amino acid sequence for mutD is shown in Figure 1. One particular mutD mutation, mutD5, is disclosed in Takano, K., et al., (1986, *Mol Gen Genet* 205, 9-13, Structure and function of dnaQ and mutD mutators of Escherichia coli). Strain CSH116 was obtained as disclosed in Miller, J. H. (1992, A Short Course in Bacterial Genetics). This strain is reported to carry the mutD5 allele. The mutD gene in this strain was found to be very different from the published mutD5. The mutD gene from strain CSH116 is designated herein as mutD5'. Table

I gives the mutations found in mutD5 and mutD5'. Further mutations in mutD which result in increased levels of mutation frequency were identified recently in Taft-Benz, S. A. et al., (1998, *Nucl. Acids Res.* 26, 4005-4011, Mutational analysis of the 3'-5' proofreading exonuclease of Escherichia coli DNA polymerase III). Table I describes various mutD mutations useful in the present invention. Table II describes various promoters used with the mutD mutations and Table III describes mutator plasmids and the range of available mutation frequencies in E.coli.

Table I mutations in the coding region of mutD

MutD				Clone		
#nucleotide	#amino acid	nucleotide	amino acid	nucleotide	amino acid	
44	15	C	Thr	T	Ile	mutD5'
218	73	T	Leu	G	Trp	mutD5
369	123	T	Thr	C	Thr	mutD5'
418	138	C	Pro	T	Pro	mutD5'
451	151	T	Ala	C	Ala	mutD5'
484	161	G	Leu	A	Arg	mutD5'
491	164	C	Ala	T	Val	mutD5
661	220	A	Glu	C	Asp	mutD5'
665	222	A	Ile	C	Leu	mutD5'
673	225	T	Ala	A	Ala	mutD5'
688	228	C	Leu	T	Leu	mutD5'
706	236	A	Lys	G	Lys	mutD5'
715	239	T	Ser	C	Ser	mutD5'
727	243	A	Arg	G	Arg	mutD5'

Table II. mutD Mutations

Name	Mutations
wild type	ATGACCGCTATG...
pOS100	**T**TGA-CGCT**T**TG...
pOS101	**G**TGACCGCT**G**TG...
pOS102	**G**TG-CCGCT**G**TG...
pOS104	**T**TGACCGCT**T**TG...
pOS105	**G**TGACCGCT**G**TGAGCACT**T(G)**CAATTACACGCCAGATCGTTCTCGATACCGAAA***T*(C)**...
pOS106	**G**TGACCGCT-TG...

C6

Table III.

Mutator (mutD5) and control (mutD) plasmids and the range of available mutation frequencies in E. coli.

#	plasmid	genotype	ori	ab resistance	size (kb)	mut. Frequency (average)	mutator rate (relative)
1	pMutD5-61	mutD5'	pSc	kan	5.97	6.4×10^{-5}	~1000-fold
2	pMutD71-Ts	mutD	pSc	kan	5.97	2.5×10^{-8}	wild type
3	pBRmutD68	mutD5'	pBR322	kan, bla	6.16	1.1×10^{-4} (AL data)	~10000-fold
4	pBRmutD727	mutD	pBR322	kan, bla	6.16	nd	wild type
Modified							
5	pOS100	mutD5'	pBR322	kan, bla	6.16	2×10^{-5}	~800-fold
6	pOS101	mutD5'	pBR322	kan, bla	6.16	3.8×10^{-6}	~152-fold
7	pOS102	mutD5'	pBR322	kan, bla	6.16	1.1×10^{-6}	~44-fold
8	pOS104	mutD5'	pSc	kan	5.97	4.35×10^{-7}	~17-fold
9	pOS105	mutD5'	pSc	kan	5.97	1.1×10^{-6}	~44-fold
10	pOS106	mutD5'	pSc	kan	5.97	5×10^{-6}	~200-fold

MutD mutations can introduce all types of base pair changes including frame shifts (Miller, supra). MutD5 has a reported relative mutation frequency of 1000-10000 fold in rich medium, ie, $5x10^{-6}$ to $5x10^{-7}$ mutations per doubling per base pair (Denegen et al., 1974,J. Bacteriol. 117, 477-478). Considering that the E.coli genome has $4.6x10^{6}$ bp, then a mutD5 gene will generate 2.3 to 23 mutations per doubling per genome. In a preferred embodiment of the present invention, mutator plasmids have been generated which allow for reduced expression levels of the mutated mutD repair gene such that the mutation rate relative to wild type is reduced. As illustrated in Table II, the MutD gene has 2 closely located ATG start codons with 6 nucleotides between them. The first ATG is considered to be putative. Both ATG codons were replaced with GTG or TTG codons for reducing mutD5' expression levels. The space between the 2 ATG codons up to 5 nucleotides was truncated. The plasmids comprising these mutator genes provide lower mutation rates when introduced in E.coli in comparison to MutD5' plasmids.

As a result, the microorganism comprising the plasmid comprising the mutated mutD gene expressed normal levels of the functional epsilon subunit coded by naturally occurring mutD and low amounts of the non-functional epsilon subunit coded by the mutated mutD5'. Both subunits compete for polymerase III. Consequently, the microorganism will most of the time have functional proof-reading but to a certain fraction of time the cell will copy its DNA without proof-reading, due to the presence of the mutD mutations. Thus, the frequency of mutagenesis of a microorganism can be altered by adjusting the expression of the mutator gene or by altering the ratio of a naturally occurring DNA repair gene to the corresponding mutated DNA repair gene. The mutations caused by these plasmid introduced mutator genes should still be as random as mutations caused by a chromosomal copy of mutD5. Data generated in Example III indicate that mutation rates of 5-1000 times over wild type are preferred for most applications. Other means of controlling the mutation frequency include having two copies of mutD on the plasmid or integrated into the microrganism genome and/or using a transmissible heat sensitive plasmid which could be used to temporarily transform cells into mutators and then restore them to wild type rates. Yet another way to adjust the mutation frequency is to identify mutD mutations which result in moderate mutation frequency due to reduced proof reading. Such mutants have been recently identified but it is not known if these mutations preferentially result in a few types of specific mutations, Taft-Benz et al., 1998, Nucl. Acids. Res. 26:4005-4011. Mutation rates are

determined using rifampicin or streptomycin as disclosed in Horiuchi, et al., 1978, Mol. Gen. Genet. 163:227-283.

Mutation rates and a description of the molecular fingerprint of a microorganism produced by the methods disclosed herein and claimed are also exemplified by virtue of the microorganism deposits made with the ATCC under the terms of the Budapest treaty.

III. Construction of mutator genes and mutator plasmids

Construction of plasmids comprising mutator genes and transformations of microorganisms can be performed by means deemed to be routine to the skilled artisan. In one embodiment illustrated herein, nucleic acid encoding a mutator gene is introduced into a microorganism on a replicating plasmid, ie, a mutator plasmid, which is cured or otherwise eliminated from the microorganism after evolution. In another embodiment disclosed herein, nucleic acid encoding a mutator gene is introduced into a microorganism's genome in addition to or as a replacement of a naturally occurring DNA repair gene.

Nucleic acid encoding a mutator gene can be isolated from a naturally occurring source or chemically synthesized as can nucleic acid encoding a protein or enzyme. Sources for obtaining nucleic acid encoding DNA repair genes mutD, mutT, mutY, mutM, mutH, mutL, mutS or mutU is provided in Section II. Figure 1 provides the nucleic acid and amino acid sequence for mutD and Table I and III provide preferred mutations for mutD and the mutation rates obtained for each construct. Once nucleic acid encoding a mutator gene is obtained, plasmids or other expression vectors comprising the mutator gene may be constructed using techniques well known in the art. Molecular biology techniques are disclosed in Sambrook et al., Molecular Biology Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and Brown, T. Current Protocols in Molecular Biology, Supplements 21, 24, 26 and 29. Nucleic acid encoding a mutator gene is obtained and transformed into a host cell using appropriate vectors. A variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression in bacteria are known by those of skill in the art.

Typically, the plasmid vector contains sequences directing transcription and translation of the nucleic acid, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a

region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. These control regions may be derived from genes homologous or heterologous to the host as long as the control region selected is able to function in the host cell.

Initiation control regions or promoters, which are useful to drive expression of the mutator gene. Virtually any promoter capable of driving expression is suitable for the present invention. Once suitable cassettes are constructed they are used to transform the host cell. General transformation procedures are taught in Current Protocols In Molecular Biology (Vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc., 1987, Chapter 9) and include calcium phosphate methods, transformation using PEG, electroporation and protoplast transformation.

After subjecting a microorganism to directed evolution using a mutator plasmid, the microorganism is cured of the mutator plasmid in order to restore the microorganism to wild-type mutation rates. Methods for curing a microorganism of a resident plasmid comprising a mutator gene include transformation of the microorganism comprising a mutator plasmid with an incompatible plasmid; electroporation techniques as described in Heery et al., 1989, Nucl. Acids. Res., 17: 10131; growth with acridine orange or ethidium bromide in the medium (Jeffrey Miller, 1972, in Curing of Episomes from E.Coli strains with Acridine Orange from Experiments in Molecular Genetics, Cold Spring Harbor Laboratories, pg. 140). In this method, acridine orange is added to 5 ml cultures of an *Enterobacteriaceae* strain at 125 μg/ml and allowed to grow overnight at 37 °C. The following day, the cultures are plated out and individual colonies are used to prepare plasmid nucleic acid. The nucleic acid is analysed by means known to those of skill in the art to determine the presence or absence of the plasmid. For microorganisms comprising a mutator gene in their genome, techniques known to those of skill in the art can be used to restore the microorganism back to wild-type mutation rates, such as excising the mutator gene or replacing the mutator gene with the naturally occurring DNA repair gene through homologous recombination techniques.

IV. Culture conditions and selective pressure

Once a microorganism has been exposed to a mutator gene, it is cultured under conditions of desired selective pressure, such as elevated temperature, pH, salt or in the presence of a solvent, such as, for example, DMF or 1,3 propanediol.

Examples of other solvents include alcohols, diols, hydrocarbon, mineral oil, mineral oil derived products, halogenated compounds and aromatic compounds.

As the skilled artisan will appreciate, growth conditions are strain dependent. General growth conditions are disclosed in Truesdell et al., (1991, Journal of Bacteriology, 173: 6651-6656) and Sonoyama et al. (1982, Applied and Environmental Microbiology, Vol. 43, p.1064-1069). Culture media may be supplemented when selectable markers are present such as antibiotic resistance genes, including but not limited to tetracycline, ampicillin or chloramphenicol.

For the methods of the present invention, cultures may be grown aerobically or anaerobically in either liquid medium or solid medium, depending upon the microorganism and the type of selection. If cultures are grown in liquid medium, it is preferred to undergo a number of rounds of replication (20 or more) in order to allow the survivors of the selection to grow over the wild-type. If cultures are grown in solid medium, such as on an agar plate, it is preferred to have a number of repetitive platings in order to pick the survivors directly and to apply higher selection pressure in each round and to amplify the population that is able to grow under the specific conditions of selection.

The manner and method of carrying out the present invention may be more fully understood by those of skill in the art by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

Examples

Example 1: Construction of *mutD* and *mutD5'* plasmids and testing in 3 bacterial strains.

The following example illustrates the construction of plasmids comprising the mutator gene, mutD5'.

mutD and *mutD5'* genes were amplified by PCR using mutd1 (5'-CGCCTCCAGCGCGACAATAGCGGCCATC-3') and mutd2 (5'-CCGACTGAACTACCGCTCCGCGTTGTG-3') primers from genomic DNA *of E. coli* and *E. coli* CSH116 (Miller 1992), respectively. The PCR products were cloned into pCR-Blunt vector (Invitrogen, Carlsbad, CA). Plasmids from clones with the correct orientation were isolated and digested with *Sma*I-*Hind*III restriction enzymes. The overhang ends were filled using T4 polymerase and cloned into pMAK705

plasmid digested with *Sma*I-*Pvu*II. The ligation products were transformed into JM101 competent cells. The resulted plasmids had the temperature-sensitive origin of replication, carried kanamycin resistance marker and were named pMutD-71 (control plasmid, wild type genotype) and pMutD5-61 (mutator plasmid).

The plasmids were successfully tested in *E. coli* MM294 (F⁻*endA1 hsdR17* (r_k^- m_k^+)*supE44 thi-1 relA1*) and *E. blattae* ATCC accession number 33429 for evolution of solvent tolerance. All evolution experiments were performed in LB medium. Mutation rates were determined by plating aliquots of cell suspensions on LB plates containing 100 ug/ml rifampicin or streptomycin. The mutation frequency was calculated by dividing the number of resistant cells by the total number of plated cells.

Example 2: Evolution of solvent tolerance.

The following example illustrates the evolution of solvent tolerant microorganisms using the mutator plasmids constructed as in Example 1.

In order to make evolution experiments quantitative, LB agar plates supplemented with 50, 60, 70, 80 and 90 g/l DMF and 25 ug/ml kanamycin were used. The size of every evolving population was limited to 10^6 cells. After each plating, the number of raised colonies was counted and 10 were selected for the next plating. Cells from selected colonies were mixed together and aliquots containing 10^6 cells were plated on fresh plates containing the same and higher concentrations of DMF. After 2 consequent platings the cells were cured of the plasmids by growth at elevated temperatures. *E. blattae* 33429 and *E. coli* MM294 were cured at 41^0 C and 43^0C, respectively. Three to four subculturings at indicated temperatures were sufficient for 87-100% curing. Individual cured clones were selected by parallel growth of clones in selective and non-selective medium. The curing was also confirmed by plasmid purification from selected clones and gel analysis.

The cured strains were tested for growth with the same DMF concentrations as their plasmid containing parents. The experiment demonstrates (1) the advantage of strains harboring mutator plasmids over strains carrying control plasmids and (2) the preservation of evolved features in strains cured from mutator plasmids.

The results of the short-term evolution are summarized in Table IV. In the process of 2 platings we obtained *E. coli* colonies, which were able to grow on plates containing 20 g/l higher concentration of DMF than control clones. Analysis of *E. coli*

MM294 harboring control and mutator plasmids revealed that the mutation frequency of cells carrying control plasmids was more then 3 orders of magnitude lower in comparison with cells containing mutator pMutD5-61. Our results showed that hypermutation was very beneficial for cell survival at elevated DMF concentrations (Table V). *E. blattae* 33429 appeared to be more sensitive to DMF. Population of 10^6 cells raised 968 colonies during 2 plating. When 10 bigger colonies were mixed and a new aliquots of 10^6 cells were plated on DMF plates supplemented with 70 g/l DMF, more than 1000 tiny colonies grew on the plates. However, these small colonies were not viable after transfer on fresh 70 g/l DMF plates. The mutation frequency of *E. blattae* 33429(pMutD5-61) dropped from 4.55×10^{-6} to 1.1×10^{-7} after second plating on plates containing 60 g/l DMF (Table V). Plasmid MutD5-61 initially provided lower mutation frequency in *E. blattae* in comparison with *E. coli*. The E.blatte strain distinctly reduced mutability after cultivation in the presence of DMF. Although E. coli and E. blattae strains belong to the family *Enterobacteriaceae*, the behavior of *E. coli mutD5* gene product could be somewhat different in *E. blattae* cell environment. Nevertheless, the benefits of pMutD5 for survival of *E. blattae* on 60 g/l DMF plates were obvious. Control cells carrying pMutD-71 couldn't grow in the presence of DMF above 50 g/l.

Contrary to *E. blattae* 33429(pMutD5-61), we did not observed significant adjustments of mutability in *E. coli* strains. The mutation frequency stayed within the same range at the end of evolution experiment. (Table V).

Single colonies of evolved cultures were used for curing experiments. The curing efficiency was 87-100 % with *E. blattae* 33429 and *E. coli* MM294. The mutation frequencies of cured clones were similar to wild type control frequencies, and cured clones preserved their ability to grow at elevated DMF concentration. *E. blattae* 33429 cured clones grew with 60 g/l DMF and *E. coli* MM294 grew with 80 g/l DMF. Initial tolerance of MM294 and E.blattae 33429 was 60 g/l and 50 g/l DMF respectively. The evolved strains increased their tolerance by 20 g/l and 10 g/l DMF, respectively. Therefore, sensitivity to DMF is strain dependent.

The advantage of mutator plasmids for evolution in liquid culture was tested as well. Within 4 days of solvent tolerance evolution in liquid medium supplemented with DMF or ethanol, *E. blattae* 33429(pMutD5-61) demonstrated growth at higher concentrations of both solvents in comparison with control cultures.

Mutator plasmids can be applied for evolution of bacterial tolerance to different solvents, various environmental stress and potentially toxic specialty

chemicals of industrial biotechnology. One advantage of the directed evolution methods disclosed herein is that the evolution of microorganisms carrying mutator plasmids can be stopped at any time. Mutator plasmids can be cured from evolving strains, and therefore, evolved desired features of the whole strain can be preserved.

TABLE IV. Evolution of solvent tolerance. Colony formation by resistant clones on LB plates supplemented with various DMF concentrations.

Strain	Genotype	DMF(g/l)	Plating 1 Number of colonies*	Plating 2 Number of colonies*
MM294(pMutD5-61)	Mutator	60 g/l	low density lawn	high density lawn
MM294(pMutD5-61)	Mutator	70 g/l	11	824
MM294(pMutD5-61)	Mutator	80 g/l	0	4
MM294(pMutD-71)	Wild type	60 g/l	17	low density lawn
MM294(pMutD-71)	Wild type	70 g/l	0	0
EB33429 (pMutD5-61)	Mutator	50 g/l	low density lawn	high density lawn
EB33429 (pMutD5-61)	Mutator	60 g/l	0	968
EB33429 (pMutD-71)	Wild type	50 g/l	793	high density lawn
EB33429 (pMutD-71)	Wild type	60 g/l	0	0

* The number of colonies represents survivors from 10^6 cells plated on LB-DMF plates.

TABLE V. Mutation frequencies of bacteria harboring mutator and control plasmids.

Strain	Mutation rate before the evolution	Mutation rate DMF (g/l)*	after the evolution
MM294(pMutD5-61)	$9.2 \pm 6.5 \times 10^{-5}$	80 g/l	$4.7 \pm 4 \times 10^{-5}$
MM294(pMutD-71)	$4.15 \pm 3.4 \times 10^{-8}$	60g/l	$2.9 \pm 2.4 \times 10^{-8}$
EB33429(pMutD5-61)	$4.55 \pm 3.7 \times 10^{-6}$	60 g/l	$1.13 \pm 0.9 \times 10^{-7}$
EB33429(pMutD-71)	$2.6 \pm 2.1 \times 10^{-8}$	50 g/l	$4.7 \pm 3.8 \times 10^{-8}$

* Single colonies from LB-DMF plates were grown in LB medium to OD A_{620}=0.8-1.2. and plated on LB-Rifampicin or Streptomycin plates at 30^0C. The experiments were done in triplicates.

Example 3: Evolution of high temperature strains.

Example 3 illustrates high temperature evolution under conditions of continuous fermentation in the mode of turbidostat which allows for fermentation wherein the cell density is stabilized. Two independent experiments were run with the strains: A: W1485 (ATCC12435) (= non mutator); B: W1485/pBRmutD68 (same strain but comprises mutator plasmid). Both strains were gown in continuous culture in a turbidostat in LB medium for about 1800 doublings. The temperature was controlled by a computer based on the measured growth rate to maintain a doubling time of about 1h. Whenever the culture grew faster the temperature was raised and vice versa. The time course of both cultures is shown in Figure 4. Initially, the culture started from W1485/pBRmutD68 evolved faster than the culture started from W1485. This indicates the advantage of the mutator plasmid. However, After about 400 doublings W1485 reached the higher temperature. We also measured the mutation rate of samples taken from both evolution experiments. Table 6 shows that the starting clones differed in their mutation frequencies by a factor of 3000. However, during the evolution experiments the mutation frequencies converged to within a factor of 2. The experiment illustrates that the rate of temperature evolution slows down over the course of the experiment. It can be expected that in a wild type strain there are a small number of genes which initially limit growth at elevated temperature. Favorable mutations in these genes will lead to relatively large gains in fitness. However, with increasing temperature more and more genes can be expected to limit growth and the pace of evolution slows down. If individual

mutations result in only very small growth benefits to their carrier then the populations have to be grown for a significant number of doublings to enrich the clones carrying these mutations from the population. As a consequence the optimum mutation rate for evolution will decrease during the process of evolution. For Table VI, mutation rates were determined using rifampicin as given in Miller (1992, supra).

Table VI Mutation rates of strains and populations used for temperature evolution

Strain/population	doublings	temperature °C	mutation rate
W1485/pBRmutD68	0	45	3142.9
AL018	210	47.22	3428.6
AL019	376	47.50	2028.6
AL038	1811	48.21	1142.9
W1485	0	45	1.0
AL017	231	47.10	0.9
AL035	543	47.91	134.3
AL040	1385	48.57	514.3

Example 4: Directed evolution of E. blattae and selection in the presence of a solvent, 1,3-propanediol.

E. Blattae ATCC accession number 33429 was transformed with plasmid pMutD68 (see Table III) and cultured in media containing 1, 5, 10, 20, and 30g/l 1,3 propanediol (cultures are designated as GEBxxx where "xxx" indicates the number of transfers into fresh media). All directed evolution experiments were carried out under anaerobic conditions in defined minimal medium with glycerol as a sole carbon source. E. blattae doesn't require vitamin B_{12} for growth, nevertheless, initial experiments were performed in 2 conditions (1) with B_{12}, and (2) without B_{12} in the growth medium.

Within 18-22h GEB001 reached maximum 1030-1060 mOD (A_{650}) at all concentrations of 1,3 propanediol. Therefore, 30g/l 1,3-PD was not inhibitory for GEB001 growth. Growth rates of GEB in the presence of 50g/l were ~ ½ of growth rates in the presence of 30g/l 1,3-PD (590 mOD : 1030 mOD in 22h). The threshold of tolerance to 1,3-PD was found between 70 to 80g/l. After 10 transfers, GEB010 was able to grow in the presence of 80g/l 1,3-PD to 350 mOD within 78h. However, these cells failed to grow at 80g/l 1,3-PD concentration after next transfer.

E.blatte is known in the art to carry the enzymatic pathway for the production of 1,3 propanediol (Roth, et al., 1986, Annu. Rev. Microbiol. 50:137-181). In order to determine if E.blatte can make 1,3-propanediol in addition to the concentrations of

1,3 propanediol added to the medium, GEB011 was grown in medium supplemented with 2-^{13}C glycerol and 70g/l 1,3-PD. The supernatant was then analyzed by NMR (^{13}C) and the results indicated the formation of ~ 2.6g/l ^{13}C 1,3-PD. Therefore, GEB cells can make 1,3 propanediol in the presence of 1,3-PD.

The evolution of 1,3-propanediol resistance was faster in the presence of B12. After 2 months of evolution GEB025 (+B12) was able to grow with 95-100g/l 1,3-propanediol. After 3 months of anaerobic growth under selection in the presence of 1,3-propanediol, GEB028 (-B12) could grow in medium supplemented with 110g/l 1,3-propanediol. Analysis of aerobic growth of GEB031 on LB plates supplemented with 85, 95, 105 and 115g/l 1.3-propanediol showed that cells produce much bigger colonies in the presence of 85g/l in comparison with 105g/l. No growth was observed at 115g/l 1,3 propanediol. The results indicate that after 3 months of applying directed evolutions techniques described herein to E.blatte, the tolerance to 1,3 propanediol was increased from 75 g/l to at least 105 g/l under aerobic conditions. The plasmid was cured from the GEB031 strain by growing at 41.5 degrees. An illustrative clone, GEB031-4 was deposited with the ATCC and has accession number.

SUB B4

FOREIGN PRIORITY

Example 5: Genetic changes in evolved E.blattae.

1,3-propanediol dehydrogenase (PDD) was compared between wild type E. blattae and the evolved strain GEB031. The PDD from the evolved strain had a higher Km for 1,3- propanediol.

Materials and Methods

SUB B5

Strains - Wild type ATCC 33429, E.blattae comprising the mutant PDD as described in Example 4 and having ATCC accession number _____.

Growth - Cells were grown in a complex medium at 30C 500 ml in a 2800 ml fernbach with shaking at 225 rpm for 20 hr. The medium consists of KH2PO4, 5.4 g/L; (NH4)2SO4, 1.2 g/L; MgSO47H2O, 0.4 g/L; yeast extract, 2.0 g/L; tryptone, 2.0 g/L; and glycerol, 9.2 g/L in tap water. The pH was adjusted to 7.1 with KOH before autoclaving (Honda, et al., 1980, J. Bacteriol, 143:1458-1465).

Extract prep - Cells were harvested by centrifugation with care to avoid anaerobic conditions. Pellets were resuspended in 100 mM Tricine pH 8.2 containing 50 mM KCl and 1 mM DTT. Cells were disrupted by passage through a French pressure cell. Crude extracts were clarified by centrifugation at 20K X g for 20 min followed by 100K X g for 1 hr to yield the high speed supernatant (HSS) fraction.

Assays - the assay for PDD was performed as described by Johnson, E.A. et al., 1987, J. Bacteriol. 169:2050-2054.

Partial purification of PDD - HSS was separated on a 16 X 100 Poros 20HQ column. The buffers were A, 50 mM HEPES, pH 7.4 containing 100 uM MnCl and B, A buffer containing 500 mM KCl. The column was loaded and developed at 10 ml/min. The gradient was 10 CV wash, a linear gradient to 70% B in 10 CV, and 1 CV to 100% B. The activity was detected in the very early fractions of the gradient. Pooled column fractions of the 33429 strain were used as collected for assays after the addition of additional of DTT to 1mM. The active fractions from strain GEB031 were pooled and concentrated on a PM30 membrane and used as concentrated after the addition of additional 1mM DTT.

Strain	GD (U/mg)	PDD (U/mg)	Ratio GD/PDD
33429	0.64	0.22	2.9
GEB031	0.79	0.08	9.9

PDD Kinetics -The results are shown below.

Strain	Km (mM Propanediol)	Km (uM NAD)
33429	28	57

SUB B6

Example 6: Cloning and sequencing the 1,3-propanediol dehydrogenase genes (*dhaT)* from *E. blattae.*

The *dhaT* genes were amplified by PCR from genomic DNA from *E. blattae* as template DNA using synthetic primers (primer 1 and primer 2) based on the *K. pneumoniae dhaT* sequence and incorporating an XbaI site at the 5' end and a

BamHI site at the 3' end. The product was subcloned into pCR-Blunt II-TOPO (Invitrogen). The cloning *dhaT* were then sequenced was standard techniques. The results of the DNA sequencing are given in SEQ ID NO:1 and SEQ ID NO:2.

Primer 1
5' TCTGATACGGGATCCTCAGAATGCCTGGCGGAAAAT3 '

Primer 2
5' GCGCCGTCTAGAATTATGAGCTATCGTATGTTTGATTATCTG3'

As will be readily understood by the skilled artisan, nucleic acid sequence generated via PCR methods may comprise inadvertent errors. The present invention also encompasses nucleic acid encoding PDD obtainable from E.blattae having ATCC accession number _____.

SUB B6 CON't.

Example 7: Comparison of wild-type E.blattae (ATCC accession number 33429) and the evolved strain GEB031-4 (ATCC accession number _____.)

This example shows that E.blattae subjected to the methods of the present invention and having ATCC accession number _____can completely consume 800mM glycerol during anaerobic fermentation and does not accumulate 3-hydroxy-propionaldehyde (3HPA) and does not lose viability. In contrast, the wild-type E.blattae accumulates 50mM 3 HPA and becomes non viable after consuming only 350 mM glycerol.

The wild-type E.blattae and the evolved E.blattae were subjected to fermentation in the following medium: 75 g glycerol, 5 g $K_2HPO_4 \cdot 3H_2O$, 3 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 4 mg $CoCl_2 \cdot 2H_2O$, 2 g yeast extract, and 1 g peptone per liter water. The pH was maintained with 20% NaOH. Both fermentations were run at 30°C with a N_2 sparge and were inoculated with a stationary grown overnight preculture.

The wild-type E.blattae accumulated 3HPA and stopped growing and metabolizing glycerol. The accumulation of 3HPA was high and reached 50mM. The cell density did not change with the culture age, but viability of the cells did. Plate counts demonstrated that accumulation of 3HPA was toxic. In contrast, the evolved strain did not accumulate more than about 6mM 3HPA and did not lose viability with culture age. After the culture had consumed all of the glycerol more was added and the culture continued converting glycerol to 1,3-propanediol. See Figures 5 and 6.

All references cited herein, including patents, patent applications, sequences and publications are hereby incorporated in their entirety by reference.